VERIFICATION OF TRANSLATION

Re: JAPANESE PATENT APPLICATION NO. 2003-315797

I, Masayo SHIMIZU, of Kitahama TNK Building, 7-1, Dosho-machi 1-chome, Chuo-ku, Osaka-shi, Osaka 541-0045, Japan hereby declare that I am the translator of the document attached and certify that the following is true translation to the best of my knowledge and belief.

Signature of translator 清水昌代

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[Document Name] Claims

[Claim 1]

A modified pyrroloquinoline quinone dependent glucose dehydrogenase (PQQGDH) that has a lower action property on disaccharides and/or improved stability than that of a wild type PQQGDH.

[Claim 2]

A modified PQQGDH according to claim 1, wherein an amino acid involved in binding of glucose and/or an amino acid in the vicinity thereof have been substituted in PQQ dependent glucose dehydrogenase of SEQ ID NO: 1.

[Claim 3]

A modified PQQGDH according to claim 1, wherein an amino acid involved in binding of calcium ion and/or an amino acid in the vicinity thereof have been substituted in PQQ dependent glucose dehydrogenase of SEQ ID NO: 1.

[Claim 4]

A modified pyrroloquinoline quinone dependent glucose dehydrogenase (PQQGDH) that has higher stability than that of a wild type PQQGDH.

[Claim 5]

A gene encoding the modified PQQGDH according to any one of claims 1 to 4.

[Claim 6]

A vector comprising the gene according to claim 5.

[Claim 7]

A transformant transformed with the vector according to claim 6.

[Claim 8]

A method of producing modified PQQGDH characterized by culturing the transformant according to claim 7.

[Claim 9]

A glucose assay kit comprising the modified PQQGDH according to any one of claims 1 to 4.

[Claim 10]

A glucose sensor comprising the modified PQQGDH according to any one of claims 1 to 4.

[Claim 11]

A method for measuring glucose comprising the modified PQQGDH according to any one of claims 1 to 4.

[Claim 12]

The modified glucose dehydrogenase according to claim 1, wherein the glucose dehydrogenase is obtained by mutating the amino acid located within a radius of 15 angstroms from the active three dimensional structure of a wild type enzyme.

[Claim 13]

The modified glucose dehydrogenase according to claim 1, wherein the glucose dehydrogenase is obtained by mutating the amino acid located within a radius of 10 angstroms from OH group which binds to the carbon at position 1 of the substance in the active three dimensional structure of the wild type enzyme.

[Claim 14]

The modified glucose dehydrogenase according to claim 2, wherein the glucose dehydrogenase is obtained by mutating the amino acid located within a radius of 10 angstroms from OH group which binds to the carbon at position 2 of the substance in the

active three dimensional structure of the wild type enzyme.

[Document Name] Description

[Title of the Invention] A Modified Pyrroloquinoline Quinone (PQQ) Dependent Glucose Dehydrogenase Having Improved Substrate Specificity or Stability

[Technical Field]

[0001]

The present invention relate to a modified glucose dehydrogenase in which a substrate specificity and/or thermal stability had been improved, and particularly relates to a modified pyrroloquinoline quinine (PQQ) dependent glucose dehydrogenase (PQQGDH) using PQQ as a coenzyme, and a method for producing thereof and a glucose sensor.

The modified PQQGDH of the present invention is useful for quantitative determination of glucose in clinical laboratory tests and food analyses.

[Background Art]

[0002]

PQQGDH is glucose dehydrogenase using pyrroloquinoline quinone (PQQ) as a coenzyme, and can be used for assay of blood glucose because it catalyzes a reaction in which glucose is oxidized to produce gluconolactone. A glucose concentration in blood is a very important indicator as an important marker for diabetes in clinical diagnosis. At present, the glucose concentration in blood is primarily measured by a biosensor using glucose oxidase, but some errors have been likely observed in measured values because the reaction is affected by a dissolved oxygen concentration. PQQ dependent glucose dehydrogenase has been noticed as a new enzyme in place of this glucose oxidase.

Our group has found that *Acinetobacter baumannii* NCIMB11517 strain produces a PQQ dependent glucose dehydrogenase, cloned a gene thereof, and constructed a high expression system thereof (see Patent document 1). The PQQ dependent glucose dehydrogenase has had an issue with substrate specificity and thermal stability compared to glucose oxidase

[Patent document 1] JP HEI-11-243949 A Publication

[Disclosure of the Invention]

[Problems to be Solved by the Invention]

[0003]

The present invention has been made in the context of problems in conventional art, makes a substrate specificity and/or thermal stability of PQQGDH a problem, and relates to improvement thereof.

[Means for Solving the Problems]

[0004]

The present inventors have conducted extensive study, and finally have completed the invention. That is, the present invention is as follow.

Item 1. A modified pyrroloquinoline quinone dependent glucose dehydrogenase (PQQGDH) that has a lower action property on disaccharides and/or improved stability than that of wild type PQQGDH.

Item 2. A modified PQQGDH according to Item 1, wherein the PQQGDH has a lower action property on disaccharides than that of wild type glucose dehydrogenase.

Item 3. A modified PQQGDH according to Item 2, wherein the disaccharide is maltose.

Item 4. A modified PQQGDH according to Item 2, wherein the PQQGDH has an action property on maltose of 90% or less compared with the action property on glucose.

Item 5. A modified PQQGDH according to Item 2, wherein the PQQGDH has a greater Km value for disaccharides.

Item 6. A modified PQQGDH according to Item 5, wherein the disaccharide is maltose.

Item 7. A modified PQQGDH according to Item 5, wherein the PQQGDH has a Km value of 8 mM or more for maltose.

Item 8. A modified PQQGDH according to Item 2, wherein the PQQGDH has a greater Km value for disaccharides than the Km value for glucose.

Item 9. A modified PQQGDH according to Item 8, wherein the disaccharide is maltose.

Item 10. A modified PQQGDH according to Item 8, wherein the PQQGDH has a Km value of 1.5 times for maltose of the Km value for glucose.

Item 11. A modified PQQGDH according to Item 2, wherein an amino acid involved in binding of glucose and/or an amino acid in the vicinity thereof have been substituted in the PQQ dependent glucose dehydrogenase of SEQ ID NO: 1.

Item 12. A modified PQQGDH according to Item 2, wherein an amino acid involved in binding of calcium ion and/or an amino acid in the vicinity thereof have been substituted in the PQQ dependent glucose dehydrogenase of SEQ ID NO: 1.

Item 13. A modified PQQGDH according to Item 2, wherein an amino acid has been substituted at least at one position selected from the group consisting of positions 67, 68, 69, 76, 89, 167, 168, 169, 170, 341, 342, 343, 351, 49, 174, 188, 189, 207, 215, 245, 249, 300, 349, 129, 130 and 131 in the PQQ dependent glucose dehydrogenase of SEQ ID NO: 1.

Item 14. A modified PQQGDH according to Item 13, wherein the amino acid substitution is selected from the group consisting of Q76N, Q76E, Q76T, Q76M, Q76G, Q76K, N167E, N167L, N167G, N167T, N167S, N167A, N167M, Q168I, Q168V, Q168A, Q168C, Q168D, Q168E, Q168F, Q168G, Q168H, Q168K, Q168L, Q168M, Q168N, Q168R, Q168S, Q168W, L169D, L169S, L169W, L169Y, L169A, L169N, L169M, L169V, L169C, L169Q, L169H, L169F, L169R, L169K, L169I, L169T, A170L, A170I, A170K, A170F, A170W, A170P, K89E, K300R, S207C, N188I, T349S, K300T, L174F, K49N, S189G, F215Y, S189G, E245D, E245F, E245H, E245M, E245N, E245Q, E245V, E245C, N249G, N249A, N249E, N249Q, A351T, P67K, E68K, P67D, E68T, I69C, P67R, E68R, E129R, K130G, P131G, E129N, P131T, E129Q, K130T, P131R, E129A, K130R, P131K, E341L, M342P, A343R, A343I, E341P, M342V, E341S, M342I, A343C, M342R, A343N, L169P, L169G and L169E.

Item 15. A modified PQQGDH according to Item 13, wherein the amino acid substitution is selected from the group consisting of Q76N, Q76E, Q76T, Q76M, Q76G, Q76K, Q168I, Q168V, Q168A, Q168C, Q168D, Q168E, Q168F, Q168G, Q168H, Q168K, Q168L,

Q168M, Q168N, Q168R, Q168S, Q168W, L169A, L169V, L169H, L169K, L169D, L169S, L169N, L169G, L169C, A170L, A170I, A170K, A170F, A170W, A170P, E245F, E245H, E245M, E245N, E245Q, E245V, E245C, N249G, N249A, N249E, N249Q, (Q168A+L169G+E245D), (Q168A+L169P+E245D), (K89E+K300R), (Q168A+L169D), (Q168S+L169S), (N167E+Q168G+L169T), (N167S+Q168N+L169R), (Q168G+L169T), (N167G+Q168S+L169Y), (N167L+Q168S+L169G), (N167G+Q168S+L169S+L174F+K49N), (Q168N+L168N+S189R), (N167E+Q168G+L169A+S189G), (N167G+Q168R+L169A), (N167S+Q168G+L169A), (N167G+Q168V+L169S), (N167S+Q168V+L169S), (N167T+Q168I+L169G), (N167G+Q168W+L169N), (N167G+Q168S+L169N), (N167G+Q168S+L169V), (Q168R+L169C), (N167S+Q168L+L168G), (Q168C+L169S), (N167T+Q168N+L169K), (N167G+Q168T+L169A+S207C), (N167A+Q168A+L169P), (N167G+Q168S+L169G), (N167G+Q168G), (N167G+Q168D+L169K), (Q168P+L169G), (N167G+Q168N+L169S), (Q168S+L169G), (N188I+T349S), (N167G+Q168G+L169A+F215Y), (N167G+Q168T+L169G), (Q168G+L169V), (N167G+Q168V+L169T), (N167E+Q168N+L169A), (Q168R+L169A), (N167G+Q168R), (N167G+Q168T), (N167G+Q168T+L169Q), (Q168I+L169G+K300T), (N167G+Q168A), (N167T+Q168L+L169K), (N167M+Q168Y+L169G), (N167E+Q168S), (N167G+Q168T+L169V+S189G), (N167G+Q168G+L169C), (N167G+Q168K+L169D), (Q168A+L169D), (Q168S+E245D), (Q168S+L169S), (A351T), (N167S+Q168S+L169S), (Q168I+L169Q), (N167A+Q168S+L169S), (Q168S+L169E), (Q168A+L169G), (Q168S+L169P), (P67K+E68K), (P67R+E68R+I69C), (P67D+E68T+I69C), (E129R+K130G+P131G), (E129Q+K130T+P131R), (E129N+P131T), (E129A+K130R+P131K), (E341L+M342P+A343R), (E341S+M342I), A343I, (E341P+M342V+A343C), (E341P+M342V+A343R), (E341L+M342R+A343N), (Q168A+L169A), (Q168A+L169C), (Q168A+L169E), (Q168A+L169F), (Q168A+L169H), (Q168A+L169I), (Q168A+L169K), (Q168A+L169M), (Q168A+L169N), (Q168A+L169P), (Q168A+L169Q), (Q168A+L169R), (Q168A+L169S), (Q168A+L169T), (Q168A+L169V), (Q168A+L169W) and (Q168A+L169Y); and has an improved substrate specificity resulting from the amino acid substitution.

Item 16. A modifid PQQGDH according to Item 2, wherein

an amino acid has been inserted between positions 428 and 429 in the PQQ dependent glucose dehydrogenase of SEQ ID NO: 1.

Item 17. A gene encoding the modified PQQGDH according to any one of Items 1 to 16.

Item 18. A vector comprising the gene according to Item 17.

Item 19. A transformant transformed with the vector according to Item 18.

Item 20. A method of producing modified PQQGDH characterized by culturing the transformant according to Item 19.

Item 21. A glucose assay kit comprising the modified PQQGDH according to any one of claims 1 to 20.

Item 22. A glucose sensor comprising the modified PQQGDH according to any one of claims 1 to 20.

Item 23. A modified pyrroloquinoline quinone dependent glucose dehydrogenase (PQQGDH) that has more enhanced stability than that of wild type PQQGDH.

Item 24. A modified PQQGDH according to Item 23 that has a survival rate of the activity of 48% or more after a thermal treatment at 58°C for 30 minutes.

Item 25. A modified PQQGDH according to Item 23 that has a survival rate of the activity of 55% or more after a thermal treatment at 58°C for 30 minutes.

Item 26. A modified PQQGDH according to Item 23 that has a survival rate of the activity of 70% or more after a thermal treatment at 58°C for 30 minutes.

Item 27. A PQQGDH according to Item 23, wherein an amino acid has been substituted at least at one position selected from the group consisting of 20, 76, 89, 168, 169, 246 and 300 in the PQQGDH of SEQ ID NO: 1.

Item 28. A modifeid PQQGDH according to Item 27, wherein the amino acid substitution is selected from the group consisting of K20E, Q76M, Q76G, K89E, Q168A, Q168D, Q168E, Q168F, Q168G, Q168H, Q168M, Q168P, Q168W, Q168Y, Q168S, L169D, L169E, L169P, L169S, Q246H, K300R, Q76N, Q76T, Q76K, L169A, L169C, L169E,

L169F, L169H, L169K, L169N, L169Q, L169R, L169T, L169Y and L169G.

Item 29. A modifeid PQQGDH according to Item 28, wherein the amino acid substitution is selected from the group consisting of K20E, Q76M, Q76G, (K89E + K300R), Q168A, (Q168A + L169D), (Q168S + L169S), Q246H, Q168D, Q168E, Q168F, Q168G, Q168H, Q168M, Q168P, Q168W, Q168Y, Q168S,(Q168S + L169E), (Q168S + L169P), (Q168A+L169A), (Q168A+L169C), (Q168A+L169E), (Q168A+L169F), (Q168A+L169H), (Q168A+L169K), (Q168A+L169N), (Q168A+L169P), (Q168A+L169Q), (Q168A+L169R), (Q168A+L169T), (Q168A+L169Y) and (Q168A + L169G); and the thermal stability is improved resulting from the amino acid substitution.

Item 30. A gene encoding the modified PQQGDH according to any one of Items 23 to 29.

Item 31. A vector comprising the gene according to Item 30.

Item 32. A transformant transformed with the vector according to Item 31.

Item 33. A method of producing modified PQQGDH characterized by culturing the transformant according to Item 32.

Item 34. A glucose assay kit comprising the modified PQQGDH according to any one of Items 23 to 32.

Item 35. A glucose sensor comprising the modified PQQGDH according to any one of Item 23 to 32.

Item 36. A method of measuring glucose comprising the modified PQQGDH according to any one of Items 23 to 32.

Item 37. A glucose dehydrogenase accordint to Item 1, which is obtained by mutating an amino acid present within a radius of 15 angstroms from the active center in a wild type enzyme.

Item 38. A glucose dehydrogenase according to Item 1 that is obtained by mutating an amino acid present within a radius of 10 angstroms from the active center in a wild type enzyme.

Item 39. A modified glucose dehydrogenase according to Item 38, that has glucose as a substrate.

Item 40. A glucose dehydrogenase accordint to Item 1, that is obtained by mutating an amino acid located within a radius of 10 angstroms from an OH group which binds to a carbon at position 1 of a substrate in the three dimensional structure of a wild type enzyme.

Item 41. A modified glucose dehydrogenase according to Item 40, that has glucose as a substrate.

Item 42. A modified glucose dehydrogenase according to Item 1, that is obtained by mutating an amino acid located within a radius of 10 angstroms from an OH group which binds to a carbon at position 2 of a substrate in the three dimensional structure of a wild type enzyme.

Item 43. A modified glucose dehydrogenase according to Item 42 that has glucose as a substrate.

[Effects of the Invention]

[0005]

The modified PQQGDH according to the present invention is an enzyme which has the lower action property on disaccharides and/or has improved thermal stability than the wild type PQQGDH. By using the modified PQQGDH according to the present invention for the glucose assay kit and the glucose sensor, it is possible to analyze with higher accuracy and provide the more stable glucose assay kit and glucose sensor compared with a case of using the wild type PQQGDH.

[Best Mode for Carrying Out the Invention]

[0006]

The invention will now be described in detail.

Herein, after removing the signal sequence, aspartic acid is numbered as 1 in the amino acid sequence.

[0007]

The modified PQQGDH of the invention includes those having the lower action property on disaccharides and/or those having improved thermal stability than the wild type PQQGDH.

As the disaccharides, maltose, sucrose, lactose and cellobiose are exemplified, and in particular, maltose is

exemplified.

The action property on the disaccharide means the action to dehydrogenate disaccharides.

If the modified PQQGDH of the present invention has the lower action property on disaccharides than that of the wild type PQQGDH, it is included in the modified PQQGDH of the present invention regardless of an increased, unchanged or lowered action property on glucose.

[0008]

The modified PQQGDH of the present invention are those having the lower action property on disaccharides in the measurement of a glucose concentration compared with the case of using the wild type PQQGDH. Those having the lower action property on maltose are preferable. The action property on maltose is preferably 90% or less, more preferably 75% or less, still more preferably 60% or less, especially 40% or less of the wild type PQQGDH.

[0009]

The modified PQQGDH of the present invention can also have a larger Km value for the disaccharides than the wild type PQQGDH. Particularly, the Km value for maltose can be large. The Km value for maltose is preferably 8 mM or more, more preferably 12 mM or more and especially 20 mM or more.

[0010]

Alternatively, the modified PQQGDH of the present invention can have a larger Km value for the disaccharides than the Km value for glucose. Particularly, a modified PQQGHD with the km value for maltose being larger than the Km value for glucose is preferable. Preferably, the Km value for maltose is 1.5 times or larger and more preferably 3 times or larger than the Km value for glucose. Herein, the action property of PQQGDH on maltose means the relative ratio of the reaction speeds of glucose and a disaccharide as a substrate, particularly of glucose and maltose as the substrate.

[0011]

Improvement in the thermal stability is preferable higher than the wild type after the thermal treatment at 58°C for 30 minutes. The survival rate of the activity is preferably 48% or more, more preferably 55% or more and in particular preferably 70% or more.

[0012]

As the modified PQQGDH of the present invention having the improved substrate specificity, for example, GDH having at least one amino acid substitution at the positions of 67, 68, 69, 76, 89, 167, 168, 169, 170, 341, 342, 343, 351, 49, 174, 188, 189, 207, 215, 245, 249, 300, 349, 129, 130 and 131 in the amino acid sequence of SEQ ID NO: 1, and GDH in which the amino acid has been inserted between positions 428 and 429 are exemplified.

[0013]

The GDH which has at least one of the amino acid substitutions selected from the group consisting of Q76N, Q76E, Q76T, Q76M, Q76G, Q76K, N167E, N167L, N167G, N167T, N167S, N167A, N167M, Q168I, Q168V, Q168A, Q168C, Q168D, Q168E, Q168F, Q168G, Q168H, Q168K, Q168L, Q168M, Q168N, Q168R, Q168S, Q168W, L169D, L169S, L169W, L169Y, L169A, L169N, L169M, L169V, L169C, L169Q, L169H, L169F, L169R, L169K, L169I, L169T, A170L, A170I, A170K, A170F, A170W, A170P, K89E, K300R, S207C, N188I, T349S, K300T, L174F, K49N, S189G, F215Y, S189G, E245D, E245F, E245H, E245M, E245N, E245Q, E245V, E245C, N249G, N249A, N249E, N249Q, A351T, P67K, E68K, P67D, E68T, I69C, P67R, E68R, E129R, K130G, P131G, E129N, P131T, E129Q, K130T, P131R, E129A, K130R, P131K, E341L, M342P, A343R, A343I, E341P, M342V, E341S, M342I, A343C, M342R, A343N, L169P, L169G and L169E, and the GDH in which L, A or K has been inserted between positions 428 and 429 are preferable.

The substitution at positions 67, 68, 69, 76, 89, 167, 168, 169, 341, 342, 343, 351, 49, 174, 188, 189, 207, 215, 245, 300, 349, 129, 130, and 131 may be performed at one position or at multiple positions.

[0014]

Herein, "Q76N" means that Q (Gln) at position 76 is

substituted with N (Asn).

[0015]

Substitutions at the positions of Q76N, Q76E, Q76T, Q76M, Q76G, Q76K, Q168I, Q168V, Q168A, Q168C, Q168D, Q168E, Q168F, Q168G, Q168H, Q168K, Q168L, Q168M, Q168N, Q168R, Q168S, Q168W, L169A, L169V, L169H, L169K, L169D, L169S, L169N, L169G, L169C, A170L, A170I, A170K, A170F, A170W, A170P, E245F, E245H, E245M, E245N, E245Q, E245V, E245C, N249G, N249A, N249E, N249Q, (Q168A+L169G+E245D), (Q168A+L169P+E245D), (K89E+K300R), (Q168A+L169D), (Q168S+L169S), (N167E+Q168G+L169T), (N167S+Q168N+L169R), (Q168G+L169T), (N167G+Q168S+L169Y), (N167L+Q168S+L169G), (N167G+Q168S+L169S+L174F+K49N), (Q168N+L168N+S189R), (N167E+Q168G+L169A+S189G), (N167G+Q168R+L169A), (N167S+Q168G+L169A), (N167G+Q168V+L169S), (N167S+Q168V+L169S), (N167T+Q168I+L169G), (N167G+Q168W+L169N), (N167G+Q168S+L169N), (N167G+Q168S+L169V), (Q168R+L169C), (N167S+Q168L+L168G), (Q168C+L169S), (N167T+Q168N+L169K), (N167G+Q168T+L169A+S207C), (N167A+Q168A+L169P), (N167G+Q168S+L169G), (N167G+Q168G), (N167G+Q168D+L169K), (Q168P+L169G), (N167G+Q168N+L169S), (Q168S+L169G), (N188I+T349S), (N167G+Q168G+L169A+F215Y), (N167G+Q168T+L169G), (Q168G+L169V), (N167G+Q168V+L169T), (N167E+Q168N+L169A), (Q168R+L169A), (N167G+Q168R), (N167G+Q168T), (N167G+Q168T+L169Q), (Q168I+L169G+K300T), (N167G+Q168A), (N167T+Q168L+L169K), (N167M+Q168Y+L169G), (N167E+Q168S), (N167G+Q168T+L169V+S189G), (N167G+Q168G+L169C), (N167G+Q168K+L169D), (Q168A+L169D), (Q168S+E245D), (Q168S+L169S), (A351T), (N167S+Q168S+L169S), (Q168I+L169Q), (N167A+Q168S+L169S), (Q168S+L169E), (Q168A+L169G), (Q168S+L169P), (P67K+E68K), (P67R+E68R+I69C), (P67D+E68T+I69C), (E129R+K130G+P131G), (E129Q+K130T+P131R), (E129N+P131T), (E129A+K130R+P131K), (E341L+M342P+A343R), (E341S+M342I), A343I, (E341P+M342V+A343C), (E341P+M342V+A343R), (E341L+M342R+A343N), (Q168A+L169A), (Q168A+L169C), (Q168A+L169E), (Q168A+L169F), (Q168A+L169H), (Q168A+L169I), (Q168A+L169K), (Q168A+L169M), (Q168A+L169N), (Q168A+L169P), (Q168A+L169Q), (Q168A+L169R),

(Q168A+L169S), (Q168A+L169T), (Q168A+L169V), (Q168A+L169W) and (Q168A+L169Y), and the insertion of L, A or K between the positions 428 and 429 contribute to enhancement of substrate specificity of PQQGDH. Herein, the substrate specificity of PQQGDH means the relative ratio of the reaction speeds of glucose and a disaccharide as a substrate, particularly of glucose and maltose.

[0016]

The PQQGDH of the present invention with improved thermal stability has amino acid substition at least at one position of positions 20, 76, 89, 168, 169, 246 and 300, and preferably has an amino acid substitution selected from the group consisting of K20E, Q76M, Q76G, K89E, Q168A, Q168D, Q168E, Q168F, Q168G, Q168H, Q168M, Q168P, Q168W, Q168Y, Q168S, L169D, L169E, L169P, L169S, Q246H, K300R, Q76N, Q76T, Q76K, L169A, L169C, L169E, L169F, L169H, L169K, L169N, L169Q, L169R, L169T, L169Y and L169G. Such substition may be at one position or at multiple positions.

[0017]

Herein, "K20E" means that K (Lys) at position 20 is substituted with E (Glu).

[0018]

Particularly, amino acid substitions of K20E, Q76M, Q76G, (K89E + K300R), Q168A, (Q168A + L169D), (Q168S + L169S), Q246H, Q168D, Q168E, Q168F, Q168G, Q168H, Q168M, Q168P, Q168W, Q168Y, Q168S,(Q168S + L169E), (Q168S + L169P), (Q168A+L169A), (Q168A+L169C), (Q168A+L169E), (Q168A+L169F), (Q168A+L169H), (Q168A+L169K), (Q168A+L169N), (Q168A+L169P), (Q168A+L169Q), (Q168A+L169R), (Q168A+L169T), (Q168A+L169Y) and (Q168A + L169G) contribute to the enhancement of the thermal stability of PQQGDH.

[0019]

The wild type PQQGDHE protein to be modified of SEQ ID NO: 1 and the base sequence of SEQ ID NO: 1 are known, and described in JP HEI-11-243949 A Publication.

[0020]

The result of X-ray crystal structure analysis of the

enzyme derived from *Acinetobacter calcoaceticus* LMD79.41 strain was reported, and the conformational structure of the enzyme including the active center has been demonstrated (see Non-patent documents 1, 2, 3 and 4).

[Non-patent document 1] J. Mol. Biol., 289, 319-333 (1999)
[Non-patent document 2] PNAS, 96(21), 11787-11791 (1999)
[Non-patent document 3] The EMBO Journal, 18(19), 5187-5194 (1999)
[Non-patent document 4] Protein Science, 9, 1265-1273 (2000)

[0021]

The modified PQQGDH of the present invention includes those in which the amino acid involved in binding of glucose and/or the amino acid in the vicinity thereof have been substituted in PQQ dependent glucose dehydrogenase described in SEQ ID NO:1.

The modified PQQGDH of the present invention includes those in which the amino acid involved in binding of calcium ion and/or the amino acid in the vicinity thereof have been substituted in PQQ dependent glucose dehydrogenase described in SEQ ID NO:1.

The modified PQQGDH of the present invention also includes those obtained by mutating the amino acid located within a radius of 15 angstroms, preferably a radius of 10 angstroms from the active center in the active three dimensional structure of the wild type enzyme.

The modified PQQGDH of the present invention also includes those obtained by mutating the amino acid located within a radius of 10 angstroms from the substrate in the active three dimensional structure of the wild type enzyme. In particular, when the substrate is glucose, those obtained by mutating the amino acid located within a radius of 10 angstroms from the substrate in the active three dimensional structure of the wild type enzyme are preferable.

The modified PQQGDH of the present invention also includes those obtained by mutating the amino acid located within

a radius of 10 angstroms from an OH group which binds to a carbon at position 1 of the substrate in the active three dimensional structure of the wild type enzyme. In particular, when the substrate is glucose, those obtained by mutating the amino acid located within a radius of 10 angstroms from the substrate in the active three dimensional structure of the wild type enzyme are preferable.

The modified PQQGDH of the present invention also includes those obtained by mutating the amino acid located within a radius of 10 angstroms from the OH group which binds to the carbon at position 2 of the substrate in the active three dimensional structure of the wild type enzyme. In particular, when the substrate is glucose, those obtained by mutating the amino acid located within a radius of 10 angstroms from the substrate in the active three dimensional structure of the wild type enzyme are preferable.

[0022]

The method of producing the modified protein of the present invention is not especially limited, and it is possible to produce by the procedure shown below. To modify the amino acid sequence which configures the protein, techniques usually performed to modify genetic information are used. That is, a DNA having the genetic information of the modified protein is made by converting a particular base or by inserting or deleting a particular base in a DNA having the genetic information of the protein. Examples of specific methods to convert the base in the DNA include use of commercially available kits (Transformer Mutagenesis Kit supplied from Clonetech; EXOIII/Mung Bean Deletion Kit supplied from Stratagene; QuickChange Site Directed Mutagenesis Kit supplied from Stratagene), or utilization of a polymerase chain reaction (PCR) method.

[0023]

The produced DNA having the genetic information of the modified protein is transferred in a state ligated with a plasmid into a host microorganism, which will become a transformant

producing the modified protein. As the plasmid in this case, pBluescript, pUC18 and the like can be utilized when using *Escherichia coli* as the host microorganism. As the host microorganism, *Escherichia coli* W3110, *Escherichia coli* C600, *Escherichia coli* JM109, *Escherichia coli* DH5a and the like can be utilized. As the method of transfecting a recombinant vector into the host microorganism, for example, when the host microorganism belongs to the genus *Escherichia*, the method of transfecting the recombinant DNA in the presence of calcium ion can be employed, and further an electroporation method may be used. In addition, commercially available competent cells (e.g., Competent High JM109 supplied from Toyobo) may also be used.

[0024]

The microorganism which is the transformant obtained above can be stably produce the modified protein in a large amount by being cultured in the nutrient medium. As a culture form of the host microorganism which is the transformant, a culture condition may be selected in consideration of nutrient physical nature of the host, and a liquid culture is performed in many cases. Industrially, it is advantageous to perform an aeration stirring culture. As nutrient sources of the medium, those usually used for the culture of the microorganism are widely used. Carbon sources may be carbon compounds capable of being assimilated, and for example, glucose, sucrose, lactose, maltose, fructose, molasses, pyruvic acid and the like are used. Nitrogen sources may be nitrogen compounds capable of being utilized, and for example, peptone, meat extract, yeast extract, hydrolyzed casein, bean cake extracted with alkali and the like are used. Additionally, phosphate salts, carbonate salts, sulfate salts, salts of magnesium, calcium, potassium, manganese and zinc, particular amino acids, particular vitamins, and the like are used as needed. A culture temperature can be optionally changed in a range in which the bacteria grow and produce the modified protein, and in the case of *Escherichia coli*, the temperature is preferably about 20 to 42°C. A culture time period is slightly

different depending on the condition, and the culture may be completed at an appropriate time period by appropriately selecting the time period when the modified protein attains to a maximum yield. Typically, the time period is about 6 to 48 hours. A pH value of the medium can be optionally changed in the range in which the bacteria grow and produce the modified protein, and preferably is in the range of about pH 6.0 to 9.0.

[0025]

The culture solution containing the microbial cells which produce the modified protein in the culture can also be directly collected and utilized, but generally in accordance with the standard methods, when the modified protein is present in the culture solution, the solution containing the modified protein and the microbial cells are separated by filtration or centrifugation, and then utilized. When the modified protein is present in the microbial cells, the microbial cells are collected from the resulting culture by a procedure such as filtration and centrifugation, then the microbial cells are disrupted by a mechanical method or an enzymatic method such as lysozyme, and if necessary the modified protein is solubilized by adding a chelating agent such as EDTA and/or a surfactant to separate and collect as an aqueous solution.

[0026]

The solution containing the modified protein obtained in such a manner may be precipitated by, for example, concentration under reduced pressure, membrane concentration, salting out treatment with ammonium sulfate or sodium sulfate, or fractional precipitation with a hydrophilic organic solvent such as methanol, ethanol and acetone. Also, heating treatment and isoelectric point treatment are effective purification procedures. Subsequently, the purified modified protein can be obtained by performing gel filtration by an absorbing agent or a gel filtrating agent, absorption chromatography, ion-exchange chromatography or affinity chromatography.

[0027]

In the present invention, the positions 76, 167, 168, 170 and 245 of PQQGDH represented by SEQ ID NO:1 were focused, amino acid substitutions thereof were made, and consequently the modified PQQGDH in which the substrate specificity had been improved could be obtained. Concerning the substrate specificity, Q76K, Q168A, A170P, E245D, (Q168A+L169G+E2 45D), (Q168A+L169P+E245D), (Q168S + L169S), (Q168A + L169D), (Q168S + E245D), (Q168S + L169E), (Q168A + L169G), (Q168S + L169P), (Q168A + L169A), (Q168A + L169C), (Q168A + L169E), (Q168A + L169K), (Q168A + L169M), (Q168A + L169N), (Q168A + L169P), (Q168A + L169S) and (Q168A + L169T) are especially preferable.

[0028]

In the present invention, the positions 20, 76, 89, 168, 169, 246 and 300 of PQQGDH represented by SEQ ID NO:1 were focused, amino acid substitutions thereof were made, and consequently the modified PQQGDH in which the stability had been improved could be obtained. So far as the thermal stability is concerned, the substitutions of K20E, (K89E + K300R), Q168A, (Q168A + L169D), (Q168S + L169S), (Q168S + L169E), (Q168S + L169P), (Q168A + L169G), Q168D, Q168E, Q168F, Q168G, Q168H, Q168M, Q168P, Q168S, Q168W, Q168Y, (Q168A + L169A), (Q168A + L169C), (Q168A + L169E), (Q168A + L169F), (Q168A + L169H), (Q168A + L169K), (Q168A + L169N), (Q168A + L169P), (Q168A + L169Q), (Q168A + L169R), (Q168A + L169T), (Q168A + L169Y) and Q246H are especially desirable.

[0029]

The modified protein can take various forms such as liquid (aqueous solution, suspension), powder and freezing and drying. The freezing and drying method is not especially limited, and may be performed in accordance with the standard method. A composition comprising the enzyme of the present invention is not limited to a frozen and dried composition, and may be a solution obtained by re-dissolving the frozen and dried composition. Glucose can be measured by various forms such as glucose assay kit and glucose sensor. The purified modified protein obtained in

this way can be stabilized by the following methods.

[0030]

The modified protein can be further stabilized by making (1) one or two or more compounds selected from the group consisting of aspartic acid, glutamic acid, α-ketoglutaric acid, malic acid, α-ketogluconic acid, α-cyclodextrin and salts thereof and (2) albumin coexist in purified modified protein.

In the frozen and dried composition, the amount of PQQGDH to be contained is different depending on the origin of the enzyme, and typically is used in the range of about 5 to 50% (weight ratio) suitably. The enzyme is suitably used in the range of 100 to 2000 U/mg in terms of enzyme activity.

Salts of aspartic acid, glutamic acid, α-ketoglutaric acid, malic acid and α-ketogluconic acid include salts of sodium, potassium, ammonium, calcium and magnesium, but are not especially limited. It is preferable to add the above compounds and the salts thereof and a-cyclodextrin in the range of 1 to 90% (weight ratio). These substances may be used alone or in combination of two or more.

A buffer contained is not especially limited, and includes Tris buffer, phosphate buffer, boric buffer and Good buffer. The pH value of the buffer is adjusted in the range of about 5.0 to 9.0 depending on a purpose for the use. The amount of the buffer to be contained in the frozen and dried composition is not especially limited, and is preferably 0.1% (weight ratio) or more and especially preferably in the range of 0.1 to 30% (weight ratio).

Usable albumin includes bovine serum albumin (BSA) and ovalbumin (OVA). Especially BSA is preferable. The amount of the albumin to be contained is preferably 1 to 80% (weight ratio), and more preferably 5 to 70% (weight ratio).

The other stabilizer and the like may be further added to the composition in the range in which no especially adverse effect is given to the reaction of PQQGDH. A combination method of the stabilizer of the present invention is not especially

limited. Examples of the method include the method of combining the stabilizer in the buffer containing PQQGDH, the method of combining PQQGDH in the buffer containing the stabilizer or the method of simultaneously combining PQQGDH and the stabilizer in the buffer.

[0031]

A stabilization effect is also obtained by adding calcium ion and a particular amino acid in combination. That is, the modified protein can be stabilized by containing: (1) the calcium ion or the calcium salt; and (2) an amino acid selected from the group consisting of glutamic acid, glutamine and lysine.

As the calcium salt, calcium salts of inorganic acids and organic acids such as calcium chloride or calcium acetate or calcium citrate are exemplified. It is preferable that the amount of the calcium ion to be contained is 1×10^{-4} to 1×10^{-2} M in the aqueous composition. Some stabilization effect can be seen when only the calcium ion or the calcium salt is contained, however, further containing the amino acid below enhances stability more.

The amino acids selected from the group consisting of glutamic acid, glutamine and lysine may be one or two or more. It is preferable that the amount of the contained amino acid selected from the group consisting of glutamic acid, glutamine and lysine is 0.01 to 0.2% by weight in the above aqueous composition.

Serum albumin may be further contained. When serum albumin is added to the above aqueous composition, it is preferable that the amount to be contained is 0.05 to 0.5% by weight.

The common buffer is used as the buffer, and it is preferable to typically make pH of the composition 5 to 10. Specifically, Tris hydrochloride buffer, boric buffer or Good buffer is used, and all buffers which do not form an insoluble salt with calcium can be used.

Other ingredients, e.g., a surfactant, a stabilizer, an excipient and the like may be added to the above aqueous

composition as needed.

[0032]

In the present invention, glucose can be measured by the following various methods.

Glucose assay kit

The present invention is characterized by the glucose assay kit comprising the modified PQQGDH according to the present invention. The glucose assay kit of the present invention contains the modified PQQGDH according to the present invention in the amount enough to assay at least once. Typically, the kit contains the buffer required for the assay, a mediator, glucose standard solutions for making a calibration curve and instructions for the use in addition to the modified PQQGDH of the present invention. The modified PQQGDH according to the present invention can be provided in various forms, e.g., as a frozen and dried reagent or a solution in an appropriate storage solution. Preferably, the modified PQQGDH of the present invention is provided as a holoenzyme, but can be provided as an apoenzyme and converted into the holoenzyme at use.

[0033]

Glucose sensor

The present invention is characterized by the glucose sensor comprising the modified PQQGDH according to the present invention. As an electrode, a carbon electrode, a gold electrode or a platinum electrode is used, and the enzyme of the present invention is immobilized on this electrode. As immobilization methods, there are the method of using a crosslinking reagent, the method of including in macromolecular matrix, the method of coating with a dialysis membrane, a optical crosslinking polymer, a conductive polymer, and a redox polymer. Alternatively, the enzyme may be immobilized in the polymer or absorbed/immobilized on the electrode with an electronic mediator typified by ferrocene or derivatives thereof. Or these may be used in combination. Preferably, the modified PQQGDH of the present invention is immobilized on the electrode as the holoenzyme, but

can be immobilized in the apoenzyme form and PQQ can be provided as another layer or in another solution. Typically, the modified PQQGDH of the present invention is immobilized on the carbon electrode using glutaraldehyde, and subsequently glutaraldehyde is blocked by treating with a reagent having an amine group.

[0034]

The glucose concentration can also be measured as follows. The buffer is placed in a thermostatic cell, PQQ, $CaCl_2$ and the mediator are added, and the temperature is kept constant. As the mediator, potassium ferricyanide and phenazine methosulfate can be used. As an action electrode, the electrode on which the modified PQQGDH has been immobilized is used, and a counter electrode (e.g., platinum electrode) and a reference electrode (e.g., Ag/AgCl electrode) are used. A constant voltage is applied to the carbon electrode, after a current becomes a steady state, a sample containing glucose is added and an increase of the current is measured. The glucose concentration in the sample can be calculated in accordance with the calibration curve made by the glucose solutions with standard concentrations.

[Examples]

[0035]

The present invention will be described in detail below based on Examples.

Example 1: Construction of expression plasmid of pyrroloquinoline quinone dependent glucose dehydrogenase gene

An expression plasmid pNPG5 of the wild type PQQ dependent glucose dehydrogenase was obtained by inserting a structural gene encoding PQQ dependent glucose dehydrogenase derived from *Acinetobacter baumannii* NCIMB11517 strain into a multicloning site of a vector pBluescript SK(-). A base sequence thereof and an amino acid sequence of PQQ dependent glucose dehydrogenase deduced from the base sequence are shown in SEQ ID NOS:2 and 1, respectively.

[0036]

Example 2: Preparation of mutant PQQ dependent glucose

dehydrogenase

A recombinant plasmid (pNPG5M1) encoding the mutant PQQ dependent glucose dehydrogenase in which glutamine at position 76 had been substituted with asparagine in the amino acid sequence described in SEQ ID NO:2 was acquired based on the recombinant plasmid pNPG5 comprising the wild type PQQ dependent glucose dehydrogenase gene, a synthetic oligonucleotide described in SEQ ID NO:3 and a synthetic oligonucleotide complementary thereto using Quick Change TM Site-Directed Mutagenesis Kit (supplied from Stratagene) by performing mutagenesis according to its protocol and further determining the base sequence.

A recombinant plasmid (pNPG5M2) encoding the mutant PQQ dependent glucose dehydrogenase in which glutamine at position 76 had been substituted with glutamic acid in the amino acid sequence described in SEQ ID NO:2 was acquired based on pNPG5, a synthetic oligonucleotide described in SEQ ID NO:4 and a synthetic oligonucleotide complementary thereto by performing the same way as in the above method.

A recombinant plasmid (pNPG5M3) encoding the mutant PQQ dependent glucose dehydrogenase in which glutamine at position 76 had been substituted with threonine in the amino acid sequence described in SEQ ID NO:2 was acquired based on pNPG5, a synthetic oligonucleotide described in SEQ ID NO:5 and a synthetic oligonucleotide complementary thereto by performing the same way as in the above method.

A recombinant plasmid (pNPG5M4) encoding the mutant PQQ dependent glucose dehydrogenase in which glutamine at position 76 had been substituted with methionine in the amino acid sequence described in SEQ ID NO:2 was acquired based on pNPG5, a synthetic oligonucleotide described in SEQ ID NO:6 and a synthetic oligonucleotide complementary thereto by performing the same way as in the above method.

A recombinant plasmid (pNPG5M5) encoding the mutant PQQ dependent glucose dehydrogenase in which glutamine at position 76 had been substituted with glycine in the amino acid sequence

described in SEQ ID NO:2 was acquired based on pNPG5, a synthetic oligonucleotide described in SEQ ID NO:7 and a synthetic oligonucleotide complementary thereto by performing the same way as in the above method.

A recombinant plasmid (pNPG5M6) encoding the mutant PQQ dependent glucose dehydrogenase in which glutamine at position 76 had been substituted with lysine in the amino acid sequence described in SEQ ID NO:2 was acquired based on pNPG5, a synthetic oligonucleotide described in SEQ ID NO:8 and a synthetic oligonucleotide complementary thereto by performing the same way as in the above method.

A recombinant plasmid (pNPG5M7) encoding the mutant PQQ dependent glucose dehydrogenase in which glutamine at position 168 had been substituted with isoleucine in the amino acid sequence described in SEQ ID NO:2 was acquired based on pNPG5, a synthetic oligonucleotide described in SEQ ID NO:9 and a synthetic oligonucleotide complementary thereto by performing the same way as in the above method.

A recombinant plasmid (pNPG5M8) encoding the mutant PQQ dependent glucose dehydrogenase in which glutamine at position 168 had been substituted with valine in the amino acid sequence described in SEQ ID NO:2 was acquired based on pNPG5, a synthetic oligonucleotide described in SEQ ID NO:10 and a synthetic oligonucleotide complementary thereto by performing the same way as in the above method.

A recombinant plasmid (pNPG5M9) encoding the mutant PQQ dependent glucose dehydrogenase in which glutamine at position 168 had been substituted with alanine in the amino acid sequence described in SEQ ID NO:2 was acquired based on pNPG5, a synthetic oligonucleotide described in SEQ ID NO:11 and a synthetic oligonucleotide complementary thereto by performing the same way as in the above method.

A recombinant plasmid (pNPG5M10) encoding the mutant PQQ dependent glucose dehydrogenase in which lysine at position 20 had been substituted with glutamine in the amino acid sequence

described in SEQ ID NO: 2 was acquired based on pNPG5, a synthetic oligonucleotide described in SEQ ID NO: 20 and a synthetic oligonucleotide complementary thereto by performing the same way as in the above method.

A recombinant plasmid encoding the mutant PQQ dependent glucose dehydrogenase in which lysine at position 89 had been substituted with glutamic acid in the amino acid sequence described in SEQ ID NO: 2 was acquired based on pNPG5, a synthetic oligonucleotide described in SEQ ID NO:23 and a synthetic oligonucleotide complementary thereto by performing the same way as in the above method. A recombinant plasmid (pNPG5M11) encoding the mutant PQQ dependent glucose dehydrogenase in which lysine at position 89 had been substituted with glutamic acid and lysine at position 300 had been substituted with arginine in the amino acid sequence described in SEQ ID NO: 2 was acquired further based on this plasmid, a synthetic oligonucleotide described in SEQ ID NO:24 and a synthetic oligonucleotide complementary thereto by performing the same way as in the above method.

A recombinant plasmid (pNPG5M12) encoding the mutant PQQ dependent glucose dehydrogenase in which glutamine at position 246 had been substituted with histidine in the amino acid sequence described in SEQ ID NO: 2 was acquired based on pNPG5, a synthetic oligonucleotide described in SEQ ID NO:25 and a synthetic oligonucleotide complementary thereto by performing the same way as in the above method.

A recombinant plasmid (pNPG5M13) encoding the mutant PQQ dependent glucose dehydrogenase in which glutamine at position 168 had been substituted with serine and leucine at position 169 had been substituted with serine in the amino acid sequence described in SEQ ID NO:2 was acquired based on pNPG5, a synthetic oligonucleotide described in SEQ ID NO:26 and a synthetic oligonucleotide complementary thereto by performing the same way as in the above method.

A recombinant plasmid (pNPG5M14) encoding the mutant

PQQ dependent glucose dehydrogenase in which glutamine at position 168 had been substituted with alanine and leucine at position 169 had been substituted with aspartic acid in the amino acid sequence described in SEQ ID NO:2 was acquired based on pNPG5, a synthetic oligonucleotide described in SEQ ID NO:27 and a synthetic oligonucleotide complementary thereto by performing the same way as in the above method.

A recombinant plasmid (pNPG5M15) encoding the mutant PQQ dependent glucose dehydrogenase in which glutamine at position 168 had been substituted with serine and leucine at position 169 had been substituted with glutamic acid in the amino acid sequence described in SEQ ID NO:2 was acquired based on pNPG5, a synthetic oligonucleotide described in SEQ ID NO:66 and a synthetic oligonucleotide complementary thereto by performing the same way as in the above method.

A recombinant plasmid (pNPG5M16) encoding the mutant PQQ dependent glucose dehydrogenase in which glutamine at position 168 had been substituted with serine and leucine at position 169 had been substituted with proline in the amino acid sequence described in SEQ ID NO:2 was acquired based on pNPG5, a synthetic oligonucleotide described in SEQ ID NO:67 and a synthetic oligonucleotide complementary thereto by performing the same way as in the above method.

A recombinant plasmid (pNPG5M17) encoding the mutant PQQ dependent glucose dehydrogenase in which glutamine at position 168 had been substituted with alanine and leucine at position 169 had been substituted with glycine in the amino acid sequence described in SEQ ID NO:2 was acquired based on pNPG5, a synthetic oligonucleotide described in SEQ ID NO:68 and a synthetic oligonucleotide complementary thereto by performing the same way as in the above method.

Escherichia coli competent cells (JM109 supplied from Toyobo) were transformed with each recombinant plasmid of pNPG5, pNPG5M1, pNPG5M2, pNPG5M3, pNPG5M4, pNPG5M5, pNPG5M6, pNPG5M7, pNPG5M8, pNPG5M9, pNPG5M10, pNPG5M11, pNPG5M12, pNPG5M13,

pNPG5M14, pNPG5M15, pNPG5M16 and pNPG5M17 to yield the transformants.

[0037]

Example 3: Construction of expression vector replicable in bacteria belonging to genus *Pseudomonas*

A structural gene portion of the mutant PQQ dependent glucose dehydrogenase was isolated by cleaving 5 μg of recombinant plasmid pNPG5M1 DNA obtained in Example 2 with restriction enzymes BamHI and XHoI (supplied from Toyobo). The isolated DNA and pTM33 (1 μg) cleaved with BamHI and XHoI were reacted with 1 unit of T4 DNA ligase at 16°C for 16 hours to ligate the DNA. Escherichia coli DH5a competent cells were transformed with the ligated DNA. The resulting expression plasmid was designated as pNPG6M1.

For each recombinant plasmid of pNPG5, pNPG5M2, pNPG5M3, pNPG5M4, pNPG5M5, pNPG5M6, pNPG5M7, pNPG5M8, pNPG5M9, pNPG5M10, pNPG5M11, pNPG5M12, pNPG5M13, pNPG5M14, pNPG5M15, pNPG5M16 and pNPG5M17, the expression plasmid was obtained by the same way as in the above method. The resulting expression plasmids were designated as pNPG6, pNPG6M2, pNPG6M3, pNPG6M4, pNPG6M5, pNPG6M6, pNPG6M7, pNPG6M8, pNPG6M9, pNPG6M10, pNPG6M11, pNPG6M12, pNPG6M13, pNPG6M14, pNPG6M15, pNPG6M16 and pNPG6M17.

[0038]

Example 4: Preparation of transformant from bacteria belonging to genus Pseudomonas

Pseudomonas putida TE3493 (Bikokenki No. 12298) was cultured in LBG medium (LB medium + 0.3% glycerol) at 30°C for 16 hours, and microbial cells were collected by centrifugation (12,000 rpm, 10 minutes). Ice-cooled 5 mM K-phosphate buffer (pH 7.0, 8 mL) containing 300 mM sucrose was added to these microbial cells to suspend the microbial cells. The microbial cells were collected again by centrifugation (12,000 rpm, 10 minutes). Ice-cooled 5 mM K-phosphate buffer (pH 7.0, 0.4 mL) containing 300 mM sucrose was added to these microbial cells to suspend the microbial cells.

The expression plasmid pNPG6M1 (0.5 μg) obtained in Example 3 was added to the suspension, and transformation was performed by the electroporation method. An objective transformant was obtained from colonies which had grown in the LB agar medium containing 100 μg/mL of streptomycin.

For each expression plasmid of pNPG6, pNPG6M2, pNPG6M3, pNPG6M4, pNPG6M5, pNPG6M6, pNPG6M7, pNPG6M8, pNPG6M9, pNPG6M10, pNPG6M11, pNPG6M12, pNPG6M13, pNPG6M14, pNPG6M15, pNPG6M16 and pNPG6M17, the transformants were acquired by the same way as in the above method.

[0039]

Test Example 1

Method of measuring GDH activity

Principle of measurement

D-glucose + PMS + PQQGDH → D-glucono-1,5-lactone + PMS (red)

2PMS (red) + NTB → 2PMS + diformazan

The presence of diformazan formed by reduction of nitrotetrazolium blue (NTB) by phenazine methosulfate (PMS) (red) was measured by spectrophotometry at 570 nm.

Definition of unit

One unit refers to the amount of the enzyme of PQQGDH to form 0.5 mM of diformazan per one minute under the following condition.

(3) Method

Reagent

A. Glucose solution: 0.5 M (0.9 g D-glucose, molecular weight: 180.16)/10 mL H_2O

B. PIPES-NaOH buffer pH 6.5: 50 mM (1.51 g of PIPES [molecular weight: 302.36] was suspended in 60 mL of water) was dissolved in 5 N NaOH, and 2.2 mL of 10% Triton-X100 is added. pH was adjusted to 6.5 ± 0.05 at 25°C using 5 N NaOH, and water was added to make 100 mL.)

C. PMS solution: 3.0 mM (9.19 mg of phenazine methosulfate [molecular weight: 817.65])/10 mL H_2O

D. NTB solution: 6.6 mM (53.96 mg of nitrotetrazolium

blue [molecular weight: 817.65])/10 mL H_2O

E. Enzyme dilution solution: 50 mM PIPES-NaOH buffer (pH 6.5) containing 1 mM $CaCl_2$, 0.1% Triton X100 and 0.1% BSA

Procedure

The following reaction mixture was prepared in a light shielding bottle, and stored on ice (prepared at use).

1.8 mL of D-glucose solution (A)

24.6 mL of PIPES-NaOH solution (pH 6.5) (B)

2.0 mL of PMS solution

1.0 mL of NTB solution (D)

[0040]

[Table 1]

Concentration in assay mixture	
PIPES buffer	42 mM
D-glucose	30 mM
PMS	0.20mM
NTB	0.22mM

[0041]

The reaction mixture (3.0 mL) was placed in a test tube (made from plastic), which was then preliminarily heated at 37°C for 5 minutes. The enzyme solution (0.1 mL) was added, and mixed by gently inverting.

The increase of absorbance for water at 570 nm was recorded by a spectrophotometer for 4 to 5 minutes with keeping the temperature at 37°C, and ΔOD per minute was calculated from an initial linear part of a curve (OD test).

At the same time, the same method except for adding the enzyme dilution solution (E) in place of the enzyme solution was repeated to measure a blank (ΔOD blank).

The enzyme powder was dissolved in the ice-cooled enzyme dilution solution (E) just before the assay, and diluted with the same buffer to 0.1 to 0.8 U/mL (due to adhesiveness of the enzyme, it is preferable to use the plastic tube).

The activity is calculated using the following formulae:

U/ml={ΔOD/min(ΔOD test - ΔOD blank)×Vt×df}/(20.1×1.0×Vs)

U/mg=(U/ml)×1/C

Vt: total volume (3.1 mL)

Vs: sample volume (1.0 mL)

20.1: 1/2 mM molecular absorbance coefficient of diformazan

1.0: light path length (cm)

df: dilution coefficient

C: enzyme concentration in solution (c mg/mL)

[0042]

Method of preparing holo type expression purified enzyme

Terrific broth (500 mL) was placed in a 2 L Sakaguchi flask, autoclaved at 121°C for 20 minutes, and after cooling, 100 μg/mL of streptomycin separately sterilized was added. A culture solution (5 mL) obtained by previously culturing Pseudomonas putida TE3493(pNPG6M1) in PY medium containing 100 μg/mL of streptomycin at 30°C for 24 hours was inoculated to this medium, and the aeration stirring culture was performed at 30°C for 40 hours. The PQQ dependent glucose dehydrogenase activity at the termination of the culture was about 120 U per mL of the culture solution in the above activity measurement.

The above microbial cells were collected by the centrifugation, suspended in 20 mM phosphate buffer (pH 7.0), and subsequently disrupted by sonication. Further the centrifugation was performed, and a supernatant solution was obtained as a crude enzyme solution. The resulting crude enzyme solution was separated and purified by HiTrap-SP (Amersham-Pharmacia) ion-exchange column chromatography. Then, the enzyme solution was dialyzed against 10 mM PIPES-NaOH buffer (pH 6.5), and calcium chloride was added at a final concentration of 1 mM. Finally, the separation/purification was performed by HiTrap-DEAE (Amersham-Pharmacia) ion-exchange column chromatography to obtain a purified enzyme preparation. The preparation obtained by the present method exhibited a nearly single band on SDS-PAGE.

Also for *Pseudomonas putida* TE3493 transformants transformed with pNPG6, pNPG6M2, pNPG6M3, pNPG6M4, pNPG6M5,

pNPG6M6, pNPG6M7, pNPG6M8, pNPG6M9, pNPG6M10, pNPG6M11, pNPG6M12, pNPG6M13, pNPG6M14, pNPG6M15, pNPG6M16, pNPG6M17, the purified enzyme preparations were acquired by the same way as in the above method.

Performances of the purified enzymes obtained in this way were evaluated.

[0043]

Measurement of Km value

In accordance with the above method of measuring the activity, the PQQGDH activity was measured. The Km value for glucose was measured by changing the substrate concentration in the above method of measuring the activity. The Km value for maltose was measured by replacing the glucose solution with a maltose solution in the above method of measuring the activity and changing the substrate concentration as was the case with the measurement of the Km value for glucose. Results are shown in Tables 2A, 2B, 6, 9 and 14.

[0044]

Substrate specificity

In accordance with the above method of measuring the activity, the PQQGDH activity was measured. The dehydrogenase activity value in the case of using glucose as the substrate and the dehydrogenase activity value in the case of using maltose as the substrate were measured, and when the measured value in the case of using glucose as the substrate was 100, a relative value was calculated. When the activity was measured in the case of using maltose as the substrate, 0.5 M maltose solution was prepared and used for the activity measurement. The results are shown in Tables 2A, 2B, 4, 5, 6, 8, 9, 11, 13 and 14.

[0045]

Measurement of thermal stability

Various PQQGDH were stored in the buffer (10 mM PIPES-NaOH, pH 6.5 containing 1 mM $CaCl_2$ and 1 μM PQQ) at an enzyme concentration of 5 U/mL, and an activity survival rate after heat treatment at 58°C was obtained. The results are shown in Tables

2A, 2B, 6, 9 and 14. The heat treatment was performed for 30 minutes only in the test in Table 2B, and for 20 minutes in the other tests.

[0046]

Measurement of optimal pH

The enzyme activity was measured in 50 mM phosphate buffer (pH 5.0 to 8.0) containing 0.22% Triton-X100, 50 mM acetate buffer (pH 3.0 to 6.0) containing 0.22% Triton-X100, 50 mM PIPES-NaOH buffer (pH 6.0 to 7.0) containing 0.22% Triton-X100 and 50 mM Tris hydrochloride buffer (pH 7.0 to 9.0) containing 0.22% Triton-X100. The results are shown in Fig. 1. The pH values at which the highest activity was exhibited are shown in Table 2A.

[0047]

[Table 2]

A

Mutant	specific activity	Substrate specificity	Km (Mal)	Km (Glc)	Optimal pH	Thermal stability
Q76N	49	66%	13.6	3.1	6.4	49.1%
Q76E	36	68%	13.6	3.7	5.6	42.5%
Q76T	32	84%	10.3	2.5	6.4	49.0%
Q76M	108	81%	8.7	2.2	6.4	55.3%
Q76G	32	84%	10.6	2.2	6.4	58.5%
Q76K	84	32%	29.9	7.9	6.8	48.4%
Q168I	231	69%	11.9	5.3	6.8	27.3%
Q168V	377	71%	13.0	6.4	6.4	32.2%
Q168A	333	37%	35.3	10.4	6.4	59.2%
Wild	1469	103%	4.1	6.5	6.4	46.7%

Note) Specific activity: enzyme activity (U/mL)/absorbance at 280 nm (ABS)

Km(Mal): Km value for maltose (mM)

Km(Glc): Km value for glucose (mM)

B

Mutant	Specific activity	Substrate specificity	Thermal stability
K20E	924	105%	49.7%
Q76M	108	81%	52.3%
Q76G	32	84%	55.1%
K89E + K300R	1038	81%	58.8%
Q168A	333	37%	55.8%
Q246H	686	192%	82.2%
Q168S+L169S	288	33%	73.0%
Q168A+L169D	106	18%	78.8%
Q168S+L169E	270	19%	47.0%
Q168S+L169P	460	25%	47.2%
Q168A+L169G	170	18%	78.3%
Wild type	1469	103%	43.4%

Note) Specific activity: enzyme activity (U/mL)/absorbance at 280 nm

[0048]

Confirmation of quantitative property of Q76K

The following reaction reagent containing 0.45 U/mL of Q76K was prepared

50 mM PIPES-NaOH buffer (pH 6.5)

1 mM $CaCl_2$

0.22% Triton X-100

0.4 mM PMS

0.26 mM WST-1 (water-soluble tetrazolium salt supplied from Dojindo Laboratories)

In accordance with the method of measuring the glucose amount shown below, as samples, purified water, serial dilutions in 10 levels of 100 mg/dL of standard solution and the glucose aqueous solution (600 mg/dL) were measured, and their linearity was confirmed. The results are shown in Fig. 2.

[0049]

Method of measuring glucose amount

The reagent (300 μL) was added to 3 μL of the sample, the change of absorbance for one minute from two minutes after adding the reagent was obtained, and the glucose amount in the sample was calculated based on a two point working line obtained from the purified water and the standard solution of 100 mg/dL glucose. As a measuring device, Hitachi 7150 type automatic analyzer was used, only a main wavelength of 480 nm was used for the measurement, and the measurement was performed at 37°C.

By Fig. 2, the good linearity was confirmed in the range of 0 to 600 mg/dL.

[0050]

Confirmation of action property of Q76K on maltose

The following reaction reagent containing 0.45 U/mL of Q76K was prepared

50 mM PIPES-NaOH buffer (pH 6.5)

1 mM $CaCl_2$

0.22% Triton X-100

0.4 mM PMS

0.26 mM WST-1 (supplied from Dojindo Laboratories)

The samples were prepared by adding 0, 120, 240, and 360 mg/dL of maltose to 100 or 300 mg/dL of glucose as the base. In accordance with the above method of measuring the glucose

amount, the measurement was performed. The measured value of 100 mg/dL of glucose containing no maltose made 100, and the samples containing 100 mg/dL of glucose as the base were relatively evaluated. Likewise, the measured value of 300 mg/dL of glucose containing no maltose made 100, and the samples containing 300 mg/dL of glucose as the base were relatively evaluated. The results are shown in Fig. 3.

[0051]

Confirmation of action property of Q76E on maltose

As was the case with confirmation of the action property of Q76K on maltose, the action property was evaluated using Q76E. The enzyme was added at a concentration of 0.24 U/mL. The results are shown in Fig. 4.

[0052]

Confirmation of action property of Q168V on maltose

As was the case with confirmation of the action property of Q76K on maltose, the action property was evaluated using Q168V. The enzyme was added at a concentration of 0.35 U/mL. The results are shown in Fig. 5.

[0053]

Confirmation of action property of Q168A on maltose

As was the case with confirmation of the action property of Q76K on maltose, the action property was evaluated using Q168A. The enzyme was added at a concentration of 0.6 U/mL. The results are shown in Fig. 6.

[0054]

Confirmation of action property of wild type enzyme on maltose

As was the case with confirmation of the action property of Q76K on maltose, the action property was evaluated using the wild type enzyme. The enzyme was added at a concentration of 0.1 U/mL. The results are shown in Fig. 7.

It was confirmed that the action property on maltose was lowered in Q76K, Q76E, Q168V and Q168A compared with the wild type enzyme from the results in Figs. 3, 4, 5, 6 and 7.

[0055]

Example 5: Construction of mutant library and screening

Random mutation was inserted into the region at positions 167 to 169 of the structural gene by PCR with the expression plasmid pNPG5 as a template. The PCR was performed in the solution of the composition shown in Table 3 under the condition at 98°C for 2 minutes, then of 30 cycles at 98°C for 20 seconds, 60°C for 30 seconds and 72°C for 4 minutes.

[0056]

[Table 3]

Reagent	Amount
KOD Dash DNA polymerase (2.5U/μl)	1.0μl
Template DNA	1.0μl
Forward primer (SEQ ID NO:12)	2.5μl
Reverse primer (SEQ ID NO:13)	2.5μl
10× buffer	5.0μl
2mM dNTPs	5.0μl
H_2O	33.0μl

[0057]

Escherichia coli DH5a strain was transformed with the resulting mutant library, formed each colony was inoculated in a microtiter plate to which 180 μL/well of LB medium (containing 100 μg/mL of ampicillin and 26 μM PQQ) had been dispensed, and cultured at 37°C for 24 hours. Each culture solution (50 μL) was transferred to another microtiter plate, and cultured microbial cells were disrupted by repeating the freezing and drying. Subsequently, the centrifugation (2000 rpm, 10 minutes) was performed, and the supernatant was collected. The collected supernatant was dispensed by each 10 μL in two microtiter plates. The activity was measured using the activity measuring reagent with glucose as the substrate in one microtiter plate, and the activity was measured using the activity measuring reagent with maltose as the substrate in another microtiter plate. Then the reactivity was compared. Many clones which exhibited the change of reactivity for maltose were obtained.

The clone which exhibited the change of reactivity for maltose was cultured in a test tube to which 5 mL of LB medium

(containing 100 μg/mL of ampicillin and 26 μM PQQ) had been dispensed, and confirmation experiments were performed. Consequently, many clones which exhibited the change of reactivity for maltose were obtained.

The results are shown in Table 4.

[0058]

[Table 4]

Mutation site	Action property on maltose	Mutation site	Action property on maltose
N167E+Q168G+L169T	64%	N167S+Q168N+L169R	80%
Q168G+L169T	42%	N167G+Q168S+L169Y	55%
N167L+Q168S+L169G	45%	N167G+Q168S+L169S+L174F +K49N	39%
Q168N+L169N+S189R	51%	N167E+Q168G+L169A+S189G	58%
N167G+Q168R+L169A	66%	N167S+Q168G+L169A	48%
N167G+Q168V+L169S	42%	N167S+Q168V+L169S	71%
N167T+Q168I+L169G	42%	N167G+Q168W+L169N	72%
N167G+Q168S+L169N	50%	N167G+Q168S+L169V	36%
Q168R+L169C	29%	N167S+Q168L+L169G	41%
Q168C+L169S	33%	N167T+Q168N+L169K	68%
N167G+Q168T+L169A+ S207C	24%	N167A+Q168A+L169P	63%
N167G+Q168S+L169G	34%	N167G+Q168G	46%
N167G+Q168D+L169K	35%	Q168P+L169G	23%
N167G+Q168N+L169S	59%	Q168S+L169G	22%
N188I+T349S	64%	N167G+Q168G+L169A+F215Y	32%
N167G+Q168T+L169G	28%	Q168G+L169V	43%
N167G+Q168V+L169T	43%	N167E+Q168N+L169A	52%
Q168R+L169A	72%	N167G+Q168R	23%
N167G+Q168T	69%	N167G+Q168T+L169Q	72%
Q168I+L169G+K300T	24%	N167G+Q168A	33%
N167T+Q168L+L169K	63%	N167M+Q168Y+L169G	60%
N167E+Q168S	32%	N167G+Q168T+L169V+S189G	42%
N167G+Q168G+L169C	37%	N167G+Q168K+L169D	41%
Q168A+L169D	16%	Q168S+E245D	29%
Q168S+L169S	26%	A351T	74%
N167S+Q168S+L169S	51%	Q168I+L169Q	51%
N167A+Q168S+L169S	40%	Q168A	35%
Q168S+L169P	20%	Q168A+L169G	16%
Q168S+L169E	15%		

[0059]

Likewise, the mutation was inserted into the region of

the positions 67 to 69 (forward primer: SEQ ID NO:14, reverse primer: SEQ ID NO:15), the region of the positions 129 to 131 (forward primer: SEQ ID NO:16, reverse primer: SEQ ID NO:17), and the region of the positions 341 to 343 (forward primer: SEQ ID NO:18, reverse primer: SEQ ID NO:19). The mutation was also attempted to be inserted between the positions 428 to 429 (forward primer: SEQ ID NO:20, reverse primer: SEQ ID NO:21). The results are shown in Table 5.

[0060]

[Table 5]

Region of positions 67 to 69

Mutation site	Action property on maltose	Mutation site	Action property on maltose
P67K+E68K	79%	P67R+E68R+I69C	80%
P67D+E68T+I69C	60%		

Region of positions 129 to 131

Mutation site	Action property on maltose	Mutation site	Action property on maltose
E129R+K130G+P131G	73%	E129Q+K130T+P131R	80%
E129N+P131T	67%	E129A+K130R+P131K	70%

Region of positions 341 to 343

Mutation site	Action property on maltose	Mutation site	Action property on maltose
E341L+M342P+A343R	80%	E341S+M342I	80%
A343I	45%	E341P+M342V+A343C	50%
E341P+M342V+A343R	76%	E341L+M342R+A343N	51%

Insertion between positions 428 and 429

Inserted amino acid	Action property on maltose	Inserted amino acid	Action property on maltose
L	73%	A	71%
K	79%		

[0061]

Among them, the mutants (Q168S+E245D, Q168A+L169D, Q168S+L169S, Q168S+L169E, Q168A+L169G, Q168S+L169P) in which the action property on maltose has largely lowered were selected, and the plasmids were extracted from these mutants. According to the

methods described in Examples 3 and 4, *Pseudomonas* was transformed to express the holoenzyme, and the purified enzymes were acquired and their properties were evaluated. The results are shown in Table 6.

[0062]

[Table 6]

Mutation	Specific activity	Substrate specificity	Km(Mal)	Km(Glc)	Thermal stability
Q168S+E245D	714	29%	24.3	14.4	55.5%
Q168A+L169D	106	18%	65.9	20.8	89.4%
Q168S+L169S	288	33%	55.1	14.4	83.9%
Q168S+L169P	460	25%	87.1	24.1	76.3%
Q168A+L169G	170	18%	60.4	18.6	89.5%
Q168S+L169E	270	19%	70.7	8.9	63.3%
Q168A	313	43%			64.4%
Wild type	1469	110%			59.8%

Note: specific activity: enzyme activity (U/mL)/absorbance at 280 nm

[0063]

Example 6: Effect of mutation at position Q168 on substrate specificity

According to the method described in Example 5, each mutant of Q168C, Q168D, Q168E, Q168F, Q168G, Q168H, Q168K, Q168L, Q168M, Q168N, Q168P, Q168R, Q168S, Q168T, Q168W and Q168Y was prepared. The primers used for the preparation of the mutants are shown in Table 7. The results of comparing the reactivity to maltose using a disruption solution prepared by test tube culture using prepared each mutant are shown in Table 8. Furthermore the plasmid was extracted from each mutant, according to the methods described in Examples 3 and 4, *Pseudomonas* was transformed to express the holoenzyme, and the purified enzymes were acquired and their properties were evaluated. The results are shown in Table 9.

[0064]

[Table 7]

Mutation site	Forward primer	Reverse primer
Q168C	SEQ ID NO:22	SEQ ID NO:23
Q168D	SEQ ID NO:22	SEQ ID NO:24
Q168E	SEQ ID NO:22	SEQ ID NO:25
Q168F	SEQ ID NO:22	SEQ ID NO:26
Q168G	SEQ ID NO:22	SEQ ID NO:27
Q168H	SEQ ID NO:22	SEQ ID NO:28
Q168K	SEQ ID NO:22	SEQ ID NO:29
Q168L	SEQ ID NO:22	SEQ ID NO:30
Q169M	SEQ ID NO:22	SEQ ID NO:31
Q168N	SEQ ID NO:22	SEQ ID NO:32
Q168P	SEQ ID NO:22	SEQ ID NO:33
Q168R	SEQ ID NO:22	SEQ ID NO:34
Q168S	SEQ ID NO:22	SEQ ID NO:35
Q168T	SEQ ID NO:22	SEQ ID NO:36
Q168W	SEQ ID NO:22	SEQ ID NO:37
Q168Y	SEQ ID NO:22	SEQ ID NO:38

[0065]

[Table 8]

Mutation site	Action property on maltose	Mutation site	Action property on maltose
Q168C	54%	Q169M	64%
Q168D	29%	Q168N	82%
Q168E	36%	Q168P	103%
Q168F	43%	Q168R	36%
Q168G	46%	Q168S	60%
Q168H	55%	Q168T	94%
Q168K	83%	Q168W	87%
Q168L	92%	Q168Y	93%
Wild type	104%		

[0066]

[Table 9]

Mutation	Specific activity	Substrate specificity	Km(Mal)	Km(Glc)	Thermal stability
Q168C	55	58%	20.4	10.7	18.2%
Q168D	102	46%	27.4	-	61.4%
Q168E	110	51%	4.7	8.6	75.4%
Q168F	137	52%	36.4	10.3	55.5%
Q168G	667	78%	11.1	-	78.7%
Q168H	486	58%	10.2	5.4	76.0%
Q168K	5	80%	9.6	2.2	-
Q168L	110	96%	8.6	4.3	37.1%
Q169M	190	68%	22.7	5.3	78.4%
Q168N	68	93%	3.6	4.1	-
Q168P	128	106%	3.5	5.1	82.3%
Q168R	57	60%	18.4	3.8	32.9%
Q168S	483	81%	12.5	3.7	80.1%
Q168T	11	103%	15.0	6.9	-
Q168W	287	96%	5.3	3.2	59.2%
Q168Y	297	99%	12.1	6.9	100.0%
Wild type	1285	106%	3.8	6.3	52.2%

Note: specific activity: enzyme activity (U/mL)/absorbance at 280 nm

[0067]

Example 7: Effects of mutation at position L169 on substrate specificity

According to the method described in Example 2, each mutant of L169A, L169V, L169H, L169Y, L169K, L169D, L169S, L169N, L169G and L169C was prepared. The primers used for the preparation of the mutants are shown in Table 10. The results of comparing the reactivity to maltose using a disruption solution prepared by test tube culture using prepared each mutant are shown in Table 11.

[0068]

[Table 10]

Mutation site	Forward primer	Reverse primer
L169A	SEQ ID NO:39	Synthetic oligonucleotide complementary to SEQ ID NO:39
L169V	SEQ ID NO:40	Synthetic oligonucleotide complementary to SEQ ID NO:40
L169Y	SEQ ID NO:41	Synthetic oligonucleotide complementary to SEQ ID NO:41
L169H	SEQ ID NO:42	Synthetic oligonucleotide complementary to SEQ ID NO:42
L169K	SEQ ID NO:43	Synthetic oligonucleotide complementary to SEQ ID NO:43
L169D	SEQ ID NO:44	Synthetic oligonucleotide complementary to SEQ ID NO:44
L169S	SEQ ID NO:45	Synthetic oligonucleotide complementary to SEQ ID NO:45
L169N	SEQ ID NO:46	Synthetic oligonucleotide complementary to SEQ ID NO:46
L169G	SEQ ID NO:47	Synthetic oligonucleotide complementary to SEQ ID NO:47
L169C	SEQ ID NO:48	Synthetic oligonucleotide complementary to SEQ ID NO:48

[0069]

[Table 11]

Mutation site	Action property on maltose	Mutation site	Action property on maltose
L169A	59%	L169D	38%
L169V	78%	L169S	57%
L169Y	107%	L169N	74%
L169H	85%	L169G	48%
L169K	60%	L169C	57%
Wild type	97%		

[0070]

Example 8: Effects of combination of mutation at position L169 with Q168A mutant on substrate specificity

According to the method described in Example 5, each mutant of Q168A+L169A, Q168A+L169C, Q168A+L169E, Q168A+L169F, Q168A+L169H, Q168A+L169I, Q168A+L169K, Q168A+L169M, Q168A+L169N, Q168A+L169P, Q168A+L169Q, Q168A+L169R, Q168A+L169S, Q168A+L169T, Q168A+L169V, Q168A+L169W and Q168A+L169Y was prepared. The primers used for the preparation of the mutants are shown in

Table 12. The results of comparing the reactivity to maltose using a disruption solution prepared by test tube culture using prepared each mutant are shown in Table 13. Furthermore the plasmid was extracted from each mutant, according to the methods described in Examples 3 and 4, *Pseudomonas* was transformed to express the holoenzyme, and the purified enzymes were acquired and their properties were evaluated. The results are shown in Table 14.

[0071]

[Table 12]

Mutation site	Forward primer	Reverse primer
Q168A+L169A	SEQ ID NO:12	SEQ ID NO:49
Q168A+L169C	SEQ ID NO:12	SEQ ID NO:50
Q168A+L169E	SEQ ID NO:12	SEQ ID NO:51
Q168A+L169F	SEQ ID NO:12	SEQ ID NO:52
Q168A+L169H	SEQ ID NO:12	SEQ ID NO:53
Q168A+L169I	SEQ ID NO:12	SEQ ID NO:54
Q168A+L169K	SEQ ID NO:12	SEQ ID NO:55
Q168A+L169M	SEQ ID NO:12	SEQ ID NO:56
Q168A+L169N	SEQ ID NO:12	SEQ ID NO:57
Q168A+L169P	SEQ ID NO:12	SEQ ID NO:58
Q168A+L169Q	SEQ ID NO:12	SEQ ID NO:59
Q168A+L169R	SEQ ID NO:12	SEQ ID NO:60
Q168A+L169S	SEQ ID NO:12	SEQ ID NO:61
Q168A+L169T	SEQ ID NO:12	SEQ ID NO:62
Q168A+L169V	SEQ ID NO:12	SEQ ID NO:63
Q168A+L169W	SEQ ID NO:12	SEQ ID NO:64
Q168A+L169Y	SEQ ID NO:12	SEQ ID NO:65

[0072]

[Table 13]

Mutation site	Action property on maltose	Mutation site	Action property on maltose
Q168A+L169A	19%	Q168A+L169P	24%
Q168A+L169C	7%	Q168A+L169Q	42%
Q168A+L169E	17%	Q168A+L169R	42%
Q168A+L169F	22%	Q168A+L169S	14%
Q168A+L169H	21%	Q168A+L169T	24%
Q168A+L169I	43%	Q168A+L169V	34%
Q168A+L169K	21%	Q168A+L169W	33%
Q168A+L169M	22%	Q168A+L169Y	37%
Q168A+L169N	19%	Wild type	104%

[0073]

[Table 14]

Mutation	Specific activity	Substrate specificity	Km(Mal)	Km(Glc)	Thermal stability
Q168A+L169A	154	19%	126	33.0	86.2%
Q168A+L169C	63	13%	103	35.6	100.0%
Q168A+L169E	90	19%	8.6	20.4	100.0%
Q168A+L169F	138	27%	44.7	10.4	80.4%
Q168A+L169H	70	27%	99.2	15.5	100.0%
Q168A+L169I	43	53%	12.5	6.0	28.7%
Q168A+L169K	129	20%	20.4	26.7	100.0%
Q168A+L169M	80	23%	52.3	15.6	-
Q168A+L169N	167	22%	59.1	34.5	83.5%
Q168A+L169P	377	24%	58.0	13.9	79.9%
Q168A+L169Q	117	49%	156.9	5.4	100.0%
Q168A+L169R	32	45%	59.0	9.6	100.0%
Q168A+L169S	42	24%	15.6	21.0	-
Q168A+L169T	98	23%	33.5	15.2	83.7%
Q168A+L169V	41	27%	49.1	24.7	40.4%
Q168A+L169W	91	38%	63.3	10.8	49.4%
Q168A+L169Y	31	52%	13.6	11.6	74.3%
Wild type	1285	106%	3.8	6.3	52.2%

Note: specific activity: enzyme activity (U/mL)/absorbance at 280 nm

[0074]

Example 9: Effects of mutation at position A170 on substrate specificity

According to the method described in Example 2, each mutant of A170C, A170D, A170E, A170F, A170G, A170H, A170K, A170L, A170M, A170N, A170P, A170R, A170S, A170T, A170W, A170Y, A170V, A170I and A170Q was prepared. For the preparation of each mutant, a synthetic oligonucleotide described in SEQ ID NO:69 was used as the forward primer, and a synthetic oligonucleotide complementary to SEQ ID NO:69 was used as the reverse primer. The objective mutant was acquired by screening the prepared mutant library. The results of comparing the reactivity to maltose using the disruption solution prepared by the test tube culture are shown in Table 15.

[0075]

[Table 15]

Mutation site	Action property on maltose	Mutation site	Action property on maltose
A170G	98%	A170K	87%
A170V	91%	A170R	108%
A170L	86%	A170C	92%
A170I	85%	A170M	90%
A170S	100%	A170F	82%
A170T	92%	A170Y	88%
A170D	102%	A170W	79%
A170E	103%	A170H	98%
A170N	100%	A170P	28%
A170Q	99%	Wild type	98%

[0076]

Example 10: Effects of mutation at position E245 on substrate specificity

According to the method described in Example 2, each mutant of E245C, E245D, E245A, E245F, E245G, E245H, E245K, E245L, E245M, E245N, E245P, E245R, E245S, E245T, E245W, E245Y, E245V, E245I and E245Q was prepared. For the preparation of each mutant, a synthetic oligonucleotide described in SEQ ID NO:70 was used as the forward primer, and a synthetic oligonucleotide complementary to SEQ ID NO:70 was used as the reverse primer. The objective mutant was acquired by screening the prepared mutant library. The results of comparing the reactivity to maltose using the disruption solution prepared by the test tube culture are shown in Table 16.

[0077]

[Table 16]

Mutation site	Action property on maltose	Mutation site	Action property on maltose
E245A	99%	E245Q	72%
E245D	49%	E245S	98%
E245F	64%	E245T	89%
E245H	54%	E245V	85%
E245I	114%	E245W	92%
E245K	Disappeared	E245Y	Disappeared
E245L	Disappeared	E245R	94%
E245M	69%	E245G	92%
E245N	59%	E245C	75%
E245P	Disappeared	Wild type	99%

[0078]

Example 11: Effects of mutation at position N249 on substrate specificity

According to the method described in Example 2, each mutant of N249C, N249D, N249A, N249F, N249G, N249H, N249K, N249L, N249M, N249E, N249P, N249R, N249S, N249T, N249W, N249V, N249I and N249Q was prepared. For the preparation of each mutant, a synthetic oligonucleotide described in SEQ ID NO:71 was used as the forward primer, and a synthetic oligonucleotide complementary to SEQ ID NO:71 was used as the reverse primer. The objective mutant was acquired by screening the prepared mutant library. The results of comparing the reactivity to maltose using the disruption solution prepared by the test tube culture are shown in Table 17.

[0079]

[Table 17]

Mutation site	Action property on maltose	Mutation site	Action property on maltose
N249G	82%	N249K	184%
N249A	77%	N249R	191%
N249V	157%	N249C	107%
N249L	94%	N249M	170%
N249I	137%	N249F	Disappeared
N249S	Disappeared	N249W	Disappeared
N249T	Disappeared	N249H	343%
N249D	Disappeared	N249P	Disappeared
N249E	86%	Wild type	106%
N249Q	79%		

[0080]

Example 12: Effects of combination with E245D mutant on substrate specificity

According to the method described in Example 2, each mutant of (Q168A+L169G+E245D) and (Q168A+L169P+E245D) was prepared. For the preparation of each mutant, a synthetic oligonucleotide described in SEQ ID NO:72 was used as the forward primer, and a synthetic oligonucleotide complementary to SEQ ID NO:72 was used as the reverse primer. As the template DNA, the plasmid of (Q168A+L169G) or (Q168A+L169P) obtained in Example 8 was used. For the prepared mutants, the results of comparing the reactivity to maltose using the disruption solution prepared by the test tube culture are shown in Table 18.

[0081]

[Table 18]

Mutation site	Action property on maltose	Mutation site	Action property on maltose
Q168A+L169G+E245D	14%	Q168A+L169G+E245D	18%
Wild type	104%		

[0082]

[Industrial Availavility]

According to the present invention, it is possible to obtain PQQGDH having the improved substrate specificity and/or thermal stability. This modified PQQGDH can be utilized for the glucose assay kit and the glucose sensor.

[0083]

[Brief Description of the Drawings]

Fig. 1 is a view showing measurement results of optimal pH for Q76N, Q76E, Q168I, Q168V, Q76T, Q76M, Q168A, a wild type, Q76G, and Q76K. A horizontal axis and a vertical axis represent pH and a relative activity, respectively. In the figure, black circles (Acetate) represent the results of measuring an enzyme activity in 50 mM acetate buffer (pH 3.0 to 6.0) containing 0.22% Triton-X100. Likewise, black squares (PIPES) represent the results of measuring the enzyme activity in 50 mM PIPES-NaOH buffer (pH 6.0 to 7.0) containing 0.22% Triton-X100, black triangles (K-PB) represent the results of measuring the enzyme activity in 50 mM phosphate buffer (pH 5.0 to 8.0) containing 0.22% Triton-X100, and black lozenges (Tris-HCl) represent the results of measuring the enzyme activity in 50 mM Tris hydrochloride buffer (pH 7.0 to 9.0) containing 0.22% Triton-X100. A measured value is represented as a relative value with the maximum activity as 100%.

Fig. 2 is a view showing the result of confirming a glucose quantitative property of Q76K. A horizontal axis and a vertical axis represent sequential dilution of one level and a measured value (mg/dl) of a glucose concentration.

Fig. 3 is a view showing the result of confirming an action property of Q76K on maltose. A horizontal axis represents a concentration (mg/dl) of added maltose, and a vertical axis represents relative % using a measured value when no maltose was added as 100%. In the figure, black triangles represent the cases of using a sample of 100 mg/dl glucose as a base to which maltose was added, and black lozenges represent the cases of using a sample of 300 mg/dl glucose as a base to which maltose was added.

Fig. 4 is a view showing the result of confirming an

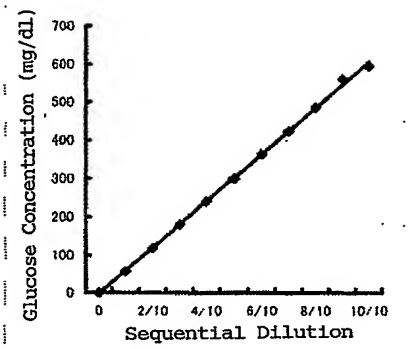
action property of Q76E on maltose. A horizontal axis represents a concentration (mg/dl) of added maltose, and a vertical axis represents relative % using a measured value when no maltose was added as 100%. In the figure, black triangles represent cases of using a sample of 100 mg/dl glucose as a base to which maltose was added, and black lozenges represent cases of using a sample of 300 mg/dl glucose as a base to which maltose was added.

Fig. 5 is a view showing the result of confirming an action property of Q168V on maltose. A horizontal axis represents a concentration (mg/dl) of added maltose, and a vertical axis represents relative % using a measured value when no maltose was added as 100%. In the figure, black triangles represent cases of using a sample of 100 mg/dl glucose as a base to which maltose was added, and black lozenges represent cases of using a sample of 300 mg/dl glucose as a base to which maltose was added.

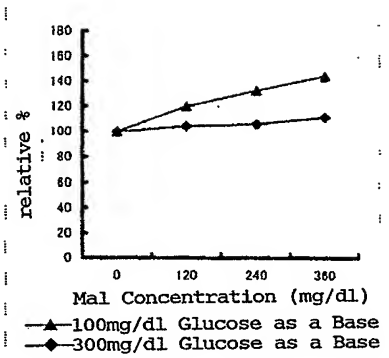
Fig. 6 is a view showing the result of confirming an action property of Q168A on maltose. A horizontal axis represents a concentration (mg/dl) of added maltose, and a vertical axis represents relative % using a measured value when no maltose was added as 100%. In the figure, black triangles represent cases of using a sample of 100 mg/dl glucose as a base to which maltose was added, and black lozenges represent cases of using a sample of 300 mg/dl glucose as a base to which maltose was added.

Fig. 7 is a view showing the result of confirming an action property of the wild type on maltose. A horizontal axis represents a concentration (mg/dl) of added maltose, and a vertical axis represents relative % using a measured value when no maltose was added as 100%. In the figure, black triangles represent cases of using a sample of 100 mg/dl glucose as a base to which maltose was added, and black lozenges represent cases of using a sample of 300 mg/dl glucose as a base to which maltose was added.

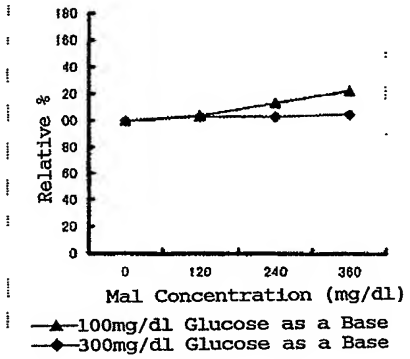
[Fig.2]



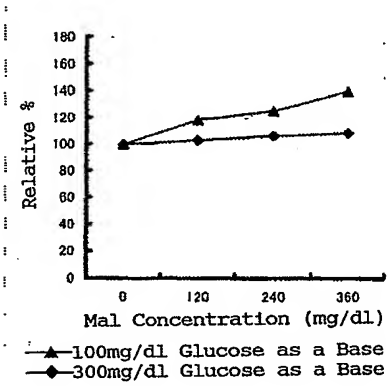
[Fig.4]



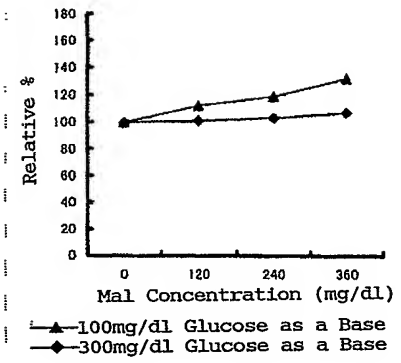
[Fig.3]



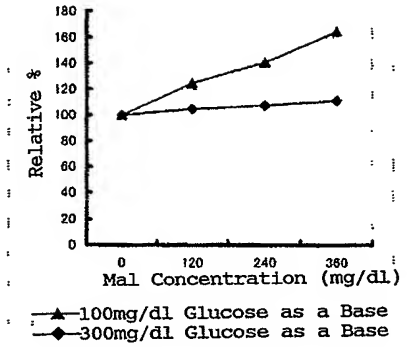
[Fig.5]



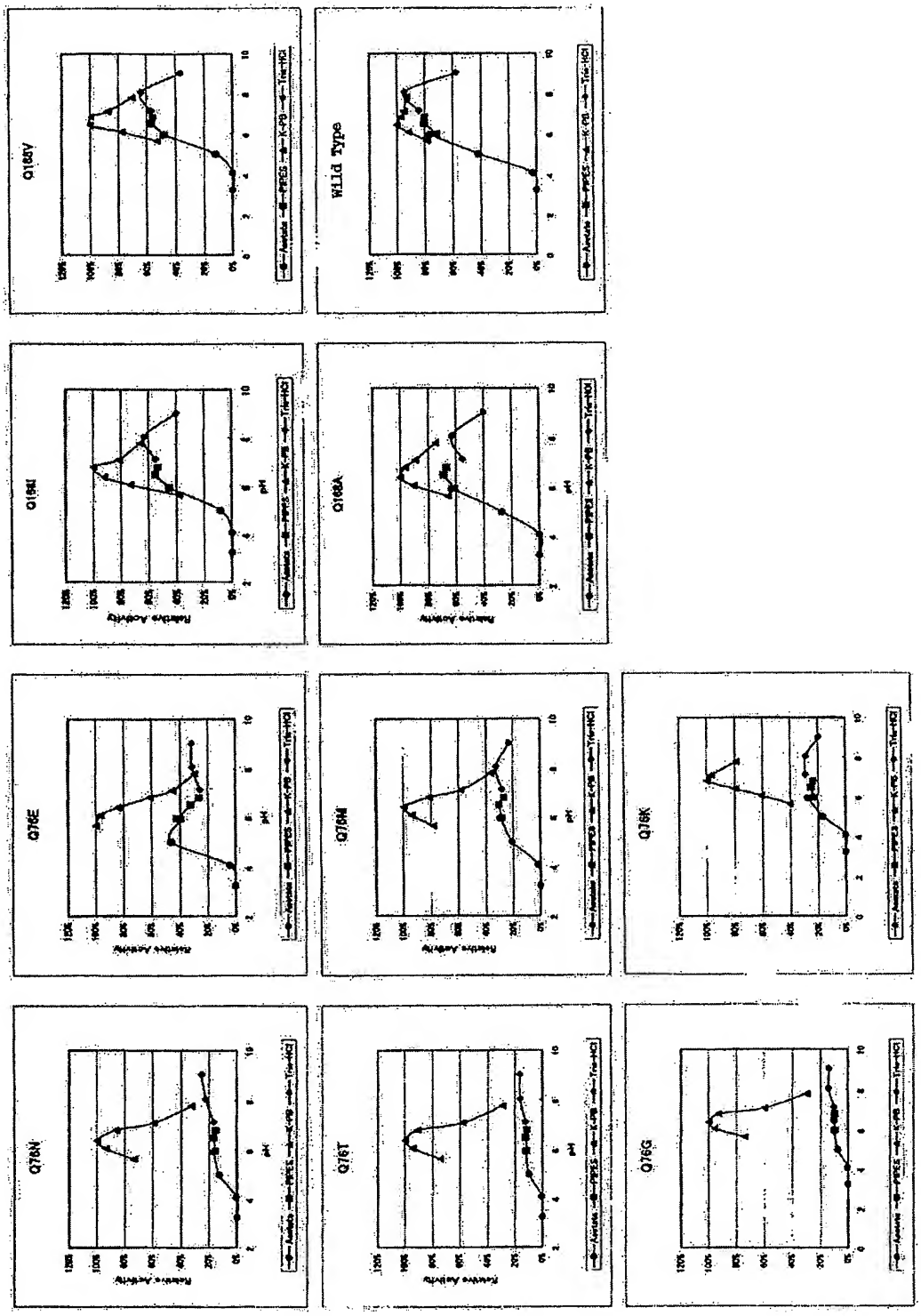
[Fig.6]



[Fig.7]


180
160
140
120
100
80
60
40
20
0
Relative %
0
120
240
360
Mal Concentration (mg/dl)
100mg/dl Glucose as a Base
300mg/dl Glucose as a Base

[Fig.1]


Q168V
Wild Type
Q168I
Q168A
Q76E
Q76M
Q76K
Q76N
Q76T
Q76G
Relative Activity
pH
Acetate
PIPES
K-PB
Tris-HCl

[Sequence Listings]

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[Document Name] Abstract

[Abstract]

[Object] An object of the present invention is to provide a PQQGDH having improved substrate specificity and/or thermal stability.

[Means for Achieving the Object] The present invention provides a modified pyrroloquinoline quinone dependent glucose dehydrogenase (PQQGDH) that has a lower action property on disaccharides than that of the wild type PQQGDH, and/or a PQQGDH that has improved thermal stability than that of the wild type PQQGDH.